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Arbeit unter wissenschaftlicher Betreuung von
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Buprenorphine via drinking water and combined oral-injection protocols for pain relief in mice

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

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Zürich 2016

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Accepted for publication in the journal Applied Animal Behaviour Science.

Vetsuisse Faculty University of Zurich, 2016

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Abstract

Buprenorphine is the opioid most commonly used in laboratory mice. To maintain therapeutic serum levels, repeated injections are required. Oral self-administration is an alternative but has been criticized to be unreliable. Here we analyse voluntary intake and water/injection combinations for their reliability to achieve effective drug supply. Mice were assigned to one of five groups: a) naïve (N); b) buprenorphine via water for 24 h (W); c) buprenorphine via two injections during light, and via water during dark phase (IW2); d) buprenorphine via three subcutaneous injections during light phase and water for 24 h (IW3) or e) surgery plus buprenorphine via three subcutaneous injections during light phase and water for 24 h (S). Drinking frequency, water and food intake, activity, body mass progression, blood serum concentrations and behavioral pain indicators were determined. Water intake was not decreased due to buprenorphine or surgery. Administration of buprenorphine resulted in an increase of activity in IW3 animals and a decrease in body mass. Food intake decreased in IW2, IW3 and S mice. All treatment groups showed mean serum concentrations higher than the targeted value throughout dark phase.
Sporadic drinking events and variable individual serum concentrations during light phase suggest the use of a combination protocol, to ensure therapeutic serum levels and minimization of pain indicators after surgery (S).

Keywords: analgesia, buprenorphine, oral administration, pain

Zusammenfassung

Buprenorphin ist eines der am häufigsten angewendeten Schmerzmittel in der Maus. Um langfristig therapeutisch wirksam zu sein, muss es repetitiv injiziert werden. Eine Alternative ist die Aufnahme über das Trinkwasser. Um diese Darreichungsform zu testen, wurden die Tiere in fünf Gruppen eingeteilt: a) naive Tiere (N); b) Buprenorphin im Trinkwasser für 24 h (W); c) zwei Buprenorphin Injektionen während der Hellphase, im Trinkwasser während der Dunkelphase (IW2); d) drei Buprenorphin Injektionen während der Hellphase, im Trinkwasser für 24 h (IW3) oder e) Operation und drei Injektionen während der Hellphase und im Trinkwasser für 24 h (S). Trinkfrequenz, Wasser- und Futteraufnahme, Aktivität, Gewichtsverlauf, Serumkonzentrationen und Schmerzindikatoren wurden erfasst. Die Wasseraufnahme wurde durch Gabe von Buprenorphin bzw. durch die Operation nicht reduziert. Buprenorphin führte zu vermehrter Aktivität und Gewichtsabnahme in der IW3 Gruppe. Buprenorphin führte zu reduzierter Futteraufnahme in der IW2, IW3 und S Gruppe. In den mit Buprenorphin behandelten Gruppen wurden während der Dunkelphase mittlere Serumkonzentrationen über dem angestrebten Wert erreicht. Wegen der sporadischen Wasseraufnahme in der Hellphase empfiehlt es sich, Injektionen in der Hellphase mit einer Buprenorphin Gabe im Trinkwasser in der Dunkelphase zu kombinieren, um eine kontinuierliche therapeutische Versorgung sicherzustellen.

Stichworte: Analgesie, Buprenorphin, orale Administration, Schmerz

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1. Introduction

Ethical, legal and scientific considerations require the effective prevention and treatment of pain in laboratory animals (Institute of Laboratory Animal Resources (U.S.) Committee on Pain and Distress in Laboratory Animals 1992).

Today, buprenorphine is one of the most widely used opioid analgesics in the treatment of pain in laboratory and companion animals (Roughan and Flecknell 2002). It is fast acting and potent, with mixed agonist-antagonist activity at classical opioid receptors and has been shown to be effective in a variety of pain models (Christoph, Kogel et al. 2005). However, to maintain therapeutically effective serum levels in mice, injections may be required more than four times in 24 h (Jirkof, Tourvieille et al. 2015).

Repeated post-surgical injections of analgesic drugs require restraint and manipulation of the animal. Handling and restraint alone may impose stress even on healthy animals (Meijer, Spruijt et al. 2006, Cinelli, Rettich et al. 2007), and are assumed to evoke additional pain, or to increase existing pain in animals with fresh surgical wounds (Jirkof, Tourvieille et al. 2015). Both the lack of efficient post-surgical pain treatment and additional handling/restraint might induce a stress response, which will have effects on physiological and endocrine function and therefore might impair the recovery of the animals. This stress response may be a significant confounder of experimental data, leading to imprecise results and therefore to increased inter- and intra-animal variation (Moberg 1999).

Attempts have been made to overcome these problems and to assure continuous and stress-free administration of buprenorphine analgesia. Several authors have described depot formulations of analgesia for rodents (Foley, Liang et al. 2011, Carbone, Lindstrom et al. 2012, Healy, Tonkin et al. 2014, Jirkof, Tourvieille et al. 2015). For example, Jirkof et al. (Jirkof, Tourvieille et al. 2015) presented a sustained release formulation of buprenorphine that offers a long-lasting, assured blood concentration, resulting in an anti-nociceptive effect, and suggested relief of post-surgical pain for 24–48 h, without causing additional stress to the animals. However, while sustained-release formulations of buprenorphine have become commercially available on the US market (Animalgesics ® for Mice, Animalgesic Labs Inc,

Millersville, MD, USA; Buprenorphine HCl CIII SR, Wildlife Pharmaceuticals Inc, Windsor, CO, USA), they are not available in Europe to date.

Oral self-administration of buprenorphine is another promising approach to administering analgesia without the negative effects of handling. Nevertheless, oral self-administration has been criticized as less effective than subcutaneous treatment in rats (Martin, Thompson et al. 2001, Thompson, Kristal et al. 2004, Thompson, DiPirro et al. 2006) and compromised bioavailability due to first-pass metabolism, referring to reduced drug concentration due to the drug being metabolized before it reaches systemic circulation, is a known obstacle in this administration route (Brewster, Humphrey et al. 1981). Despite these concerns, several studies in mice and rats have shown that buprenorphine has sufficient analgesic efficacy when administered orally. Several routes of oral administration have been described, such as mixing buprenorphine with flavored gelatin (Liles, Flecknell et al. 1998), Nutella ® (Goldkuhl, Jacobsen et al. 2010, Kalliokoski, Jacobsen et al. 2011), gel delivery systems (Hovard, Teilmann et al. 2015) or with the regular diet of the mice (Molina-Cimadevila, Segura et al. 2014).

While these routes of administration have been shown to provide analgesia, they also have their limitations. For instance, food neophobia is a well-known obstacle in the oral administration of analgesics in mice. Habituation to new food items is necessary in order to ensure sufficient intake and resulting therapeutic drug levels (Liles, Flecknell et al. 1998, Goldkuhl, Jacobsen et al. 2010, Kalliokoski, Jacobsen et al. 2011, Hovard, Teilmann et al. 2015). Moreover, even after habituation, the latency to ingestion of the drug, as well as the total amount ingested by the animals, might be difficult to anticipate (Hovard, Teilmann et al. 2015). Providing buprenorphine mixed with the regular diet might overcome the problem of food neophobia, as stated by Molina-Cimadevila et al. (Molina-Cimadevila, Segura et al. 2014). Nonetheless, medicated food items need to be prepared prior to administration, which might be costly and time consuming depending on the chosen food medium (Liles, Flecknell et al. 1998, Goldkuhl, Jacobsen et al. 2010, Kalliokoski, Jacobsen et al. 2011, Hovard, Teilmann et al. 2015). Alternatively, providing analgesia mixed with drinking water is a promising route of administration (Hayes, Raucci et al. 2000, Jessen, Christensen et al. 2007) since tap water is readily available at every facility and mixtures can be prepared within minutes.

The present study aimed to explore whether administering buprenorphine in drinking water offers a reliable treatment option for pain management in mice or if a combination with buprenorphine injections may be necessary for reliable drug supply. In a first experiment three analgesic protocols were tested: administration via drinking water (*W*), a combination of two buprenorphine injections during the light phase and administration via drinking water in the dark phase (*IW2*) and a combination of three buprenorphine injections during the light phase and administration via drinking water for 24 h (*IW3*). Drinking behaviour, spontaneous water and food intake, blood serum concentrations reached by the drug over time, and behavioural modifications possibly evoked by the drug were assessed.

We hypothesize that laboratory mice drink the buprenorphine treated water regularly and in sufficient amounts, at least during the dark phase, to reach continuous therapeutic buprenorphine serum concentrations and also to minimize pain indicators after one-side sham embryo transfer. We therefore tested in a second experiment the most promising analgesic protocol (*IW3*) for its reliability in assuring pain relief in surgically treated mice, using clinical investigation and behaviour-based pain assessment.

2. Materials and Methods

2.1 Ethics statement

The animal housing and experimental protocols were approved by the Cantonal Veterinary Office, Zurich, Switzerland, under license no. 181/2012, and were in accordance with Swiss Animal Protection Law and conform to European Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes and to the Guide for the Care and Use of Laboratory Animals (Worlein, Baker et al. 2011).

2.2 Animals and standard housing conditions

The animals were 110 female C57BL/6J mice obtained at the age of 4–5 weeks (Charles River, Sulzfeld, Germany). Their health status was monitored by a health surveillance programme according to FELASA guidelines. The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations, except for *Helicobacter* species (Mahler, Berard et al. 2015).

Mice were housed in groups of four to eight animals for three weeks prior to testing. All animals were maintained in Eurotyp III clear transparant plastic cages (Techniplast, Hohenpeissenberg, Germany) with a 12-hour light/dark cycle (lights on at 8:00) with artificial light (approximately 40 lx in the cages), controlled temperature and relative humidity of 21 ± 1C° and 55 ± 10%. They were fed a pelleted and extruded mouse diet (Kliba No. 3436, Provimi Kliba, Kaiseraugst, Switzerland) ad libitum and had unrestricted access to drinking water. Autoclaved dust-free sawdust bedding (80–90 g per cage; LTE E-001 Abedd, Indulab), autoclaved hay (8–12 g per cage; Winzeler, Affoltern am Albis, Switzerland) and one nestlet™ (5 cm x 5cm), consisting of cotton fibres (Indulab AG, Gams, Switzerland), as nesting material and cardboard shelters (Ketchum Manufacturing, Brockville, Canada) were provided.

2.3 Treatment protocols

Naïve mice (N): Naïve mice received tap water for the experimental period.

Buprenorphine administration via drinking water (W): Temgesic (Temgesic solution, 0.3mg/ml, Reckitt Benckiser, Switzerland), a water-soluble buprenorphine medicinal product, was administered in the drinking water of the mice. Temgesic was diluted using tap water to a dose of 0.009 mg/ml drinking water. At the beginning of the light phase (8:00), mice were provided with a freshly prepared bottle of buprenorphine-treated water. The dose of buprenorphine was chosen to be approximately 10 times higher than the subcutaneous dose (Liles, Flecknell et al. 1998, Roughan and Flecknell 2002), assuming that a mouse would drink approximately 3 ml of the buprenoprhine-treated water per day.

Buprenorphine administration via two injections during light phase and drinking water during dark phase (IW2): Mice were injected subcutaneously twice at a commonly used dose of 0.1 mg buprenorphine /kg body mass, at 4 h (12:00) and 10 h (18:00) after the beginning of the light phase. Shortly before the injection, Temgesic was diluted in sterile NaCL (0.9%) so that the injection volume was 2 µl/g body mass. Following the second injection, the animals were provided with buprenorphine-treated drinking water overnight, prepared as described above. Injection times were chosen dependent on the assumption that, if surgery is performed at the onset of the light phase, post-operative pain management should start 4 h later (as pre-operative administered buprenorphine loses its efficacy after 4–6 h). The second injection time was chosen to be 6 h after the first injection and, at the same time, mice were provided with buprenorphine-treated water. We assumed that mice would start to drink the buprenorphine-treated water regularly from the onset of the dark phase, 2 h after presentation.

Buprenorphine administration via three injections during light phase and drinking water for 24 h (IW3):

Mice were injected subcutaneously three times at a commonly used dose of 0.1 mg buprenorphine/kg body mass, with the beginning of the light phase (8:00) and at 4 h (12:00) and 8 h (16:00) after the beginning of the light phase. Injection times were chosen dependent on the assumption that, if surgery is performed at the onset of the light phase pre-emptive pain management should be administred before surgery. Post-operative pain management should start 4 h after first injection. The third injection time was chosen to be 4 h after the second injection. Additonally, mice were

provided with buprenorphine-treated drinking water, prepared as described above, from the beginning of the light phase (8:00) for 24 h.

Experimental design, experimental animal housing conditions and data acquisition:
Forty mice were assigned randomly to one of five groups in equal numbers (n=8) for behavioural assessment (drinking behaviour analyses, activity, food and water intake, body mass progression, with additional pain scoring in the surgery group): a) naïve mice (N), b) buprenorphine administration via drinking water (W), c) buprenorphine administration via two injections during light phase and drinking water during dark phase (IW2), d) buprenorphine administration via three injections during light phase and drinking water for 24h (IW3) or e) surgery plus buprenorphine administration via three injections during light phase and drinking water for 24h (S).
After behavioural assessment mice were allowed to recover for 2-3 weeks in standard housing conditions. Except for the surgery group, all other mice (n=32) were used additionally for blood serum sampling after the recovery period, to reduce the total number of animals used in this study.

2.4 Experiment 1

2.4.1 Behavioural assessment

Mice were housed individually in a special observation cage with raised plastic (465 mm high) walls instead of a cage grid. The observation cage had the same floor space as the standard cage and was equipped with a water bottle fixed outside of the cage with the nipple extended into the cage. Their usual diet and a nestlet were provided on the cage floor of the observation cage. Prior to experiments, mice were allowed to become accustomed to the new housing conditions for three days.

2.4.2 Drinking behavior

Behaviour was recorded digitally in the absence of a human observer with infrared sensitive cameras (Ikegami). Each cage was filmed with a single camera; cameras and infrared light sources were attached 1.5 meters above the cages. The recorded material (24 hours of continuous footage) was analyzed visually; time points of water consumption and drinking frequencies were recorded.

2.4.3 Activity analyses

Videos recorded as described above on the first day of treatment were analyzed with automated tracking software (EthovisionXT 7, Wageningen, The Netherlands). The distance (in cm) moved by the animal was assessed automatically (movement of animal`s center point was tracked) to measure static behaviours, i.e. resting, grooming etc., as well as horizontal locomotion.

2.4.4 Body mass, food and water intake

Body mass progressions, as well as food and water consumption were measured at the beginning of the light phase one day before experiment and on day one and two. Weights were obtained by using a precision balance (PR 2003, Delta Range, Mettler-Toledo AG, Greifensee, Switzerland) up to an accuracy of two decimal places.

2.4.5 Buprenorphine serum concentration

Mice were group-housed in standard cages as described above. Cages were assigned randomly to one of three experimental groups as follows:

Administration via drinking water (W): Six animals were bled at one of six time points (day 1: 12:00, 16:00, 18:00, 22:00; day 2: 2:00, 6:00).

Administration via two injections during light phase and drinking water during dark phase (IW2): Six animals were bled at one of five time points (day 1: 16:00, 18:00, 22:00; day 2: 2:00, 6:00).

Administration via three injections during light phase and drinking water for 24 h (IW3): Six animals were bled at one of six time points (Day 1: 12:00, 16:00, 20:00, 22:00, Day 2: 2:00, 6:00).

Time points were chosen depending on the buprenorphine administration protocol applied. Previous studies showed that subcutaneous injections of buprenorphine resulted in serum concentrations thought to be therapeutic for at least 4 h (4). Since W and IW3 animals were provided with buprenorphine at 8:00, the first measurement time for those groups was set at 12:00, 4 h after first presentation, respectively after first injection.

In IW2 animals, the first time point set was 16:00, 4 h after first buprenorphine injection. For all protocols, subsequent measurements were performed at intervals no longer than 4 h.

Blood was sampled by sublingual vein puncture under sevoflurane anesthesia. The amount collected was < 20% of the total blood volume of the animal (Diehl, Hull et al. 2001, Heimann, Kasermann et al. 2009, Heimann, Roth et al. 2010).

Blood was centrifuged and the serum stored at –20°C until further analysis. Buprenorphine serum concentrations were determined by Ultraperformance liquid chromatography tandem mass-spectrometry by a commercial laboratory, accredited for medical laboratory diagnostics according to DIN EN ISO 15189:2007 (MVZ Labor Dessau GmbH, Germany).

2.5 Experiment 2

2.5.1 Behavioural assessment

Drinking behaviour, home cage activity, food and water intake, as well as body mass progression, were monitored analogous to experiment 1.

After evaluation of data from experiment one, we assumed that pain relief could be assured using IW3 as the analgesic protocol for the surgery group.

2.5.2 Surgical procedure

The experiment began with a subcutaneous injection of buprenorphine with the beginning of the light phase (8:00). Forty-five minutes after injection, animals were transferred in transport cages (i.e. standard cages with filter top) to a nearby operating theatre. Mice were anaesthetized with sevoflurane (Sevorane, Abbott, Baar, Switzerland) as mono-anaesthesia. The anaesthetic gas was provided with a rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland); oxygen was used as carrier gas. After induction of anaesthesia in a perspex induction chamber (7–8% sevoflurane, 600 mL/min gas flow), animals were transferred to a warmed (39 ± 1◦C) operating table, and anaesthesia was maintained via nose mask. Eye ointment was applied, the fur was clipped and the operating field disinfected with ethanol (70%). Mice underwent a one-side sham embryo transfer. The incision in the abdominal muscle wall was closed with absorbable sutures (Vicryl, 6/0 polyglactin 910, Ethicon Ltd, Norderstedt, Germany), and the skin was closed using skin staples (Precise, 3M Health Care, StPaul, MN, USA). Surgery was completed within 3–4 min, anaesthesia lasted 6–8 min. While regaining consciousness animals stayed for ~10 min on the warmed table before being transferred back to the animal room. Animals were allowed to recover fully from anaesthesia in the transport cage for one hour in a warming cabinet (32°C) prior to subsequent behavioural observation.

2.5.3 Pain scoring

Animals were transferred to an observation chamber at 1, 3, 5, 7 and 24 hours after the animals had undergone surgery. After ten minutes of habituation clinical investigation and behaviour-based pain assessment lasted three minutes per animal and was performed by a trained and blinded observer. According to a routinely used scoring system documenting the general condition of an animal (Arras, Rettich et al. 2007), abnormalities of body condition (e.g. sunken flanks), fur condition (e.g. ruffled coat), eyes (e.g. discharge), breathing (e.g. irregular) and posture (e.g. hunched back) were registered, and the wound was checked. Additionally, changes in facial expression, namely orbital tightening and the ear position (Langford, Bailey et al. 2010) were judged. Symptoms were converted into scores according to a scoring system (Table 1).

2.6 Statistical analyses

Power calculation for group size determination was performed with G*Power 3.1. Statistical analyses were performed with SPSS 22 software (IBM, Armonk, NY, USA). All data were tested for normal distribution and homogeneity of variance (Shapiro-Wilks, Levene's test).

Mean and standard deviation of the mean were calculated for all parameters. One way analyses of variance (ANOVA) were performed for percentage change in body mass (% change compared with baseline measurements one day before experiments), Δ weight of food pellets and water bottles (Δ compared with measurements one day before the experiment), as well as for center point distance moved during 24 h of activity analyses, followed by post hoc tests (Bonferroni) to show significant differences between groups.

Significance for all statistical tests was established at $p \leq 0.05$.

3. Results

3.1 Experiment 1

3.1.1 Behavioural assessment

3.1.1.1 Drinking behaviour

While water intake during the dark phase was frequent and regular, intake during the light phase was rather sporadic and infrequent in all groups. The overall circadian pattern of drinking events appears unchanged in response to buprenorphine treatment (Figure 1A).

3.1.1.2 Activity analyses

Naïve animals moved a mean distance of 1284±365 cm within 24 h. Distance moved was higher in mice receiving buprenorphine treatment in all groups compared to naïve mice ($F(5, 39)=3.616$, $p=0.009$); however, these differences were only significant in *IW3* animals ($p=0.005$, Figure 2 A).

3.1.1.3 Body mass, food and water intake

Body mass: Mice weighed on average 21±1.7 g at 24 h before the start of the experiment. No significant difference in percent body mass progression between groups was found ($F(5, 42)=1.633$, $p=0.172$, Figure 2 B).

Food intake: Naïve mice ate in general 3.6±0.4 g of food pellets a day. Significant differences between groups were found ($F(5, 40)=8.763$, $p\leq0.0001$). Compared with naïve mice, food intake was decreased significantly in IW2 and IW3 animals, whereas animals which had only been provided with buprenorphine in the drinking water did not decrease their food intake significantly (W) (IW2: $p=0.001$, IW3: $p=0.023$, Figure 2 C).

Water intake: Naïve mice drank in general 4.8±0.3 g of water a day. Water intake did not differ significantly between the treatment groups ($F(5, 42) =1.851$, $p=0.124$, Figure 2D).

3.1.2 Buprenorphine serum concentration

Mean serum concentrations of buprenorphine remained high throughout the dark phase in all buprenorphine-treated groups (Table 2). In W and IW3 animals, mean serum concentrations remained high throughout the entire investigation period, while IW2 animals showed constant high serum concentrations only during the dark phase. Individual comparison of serum concentrations revealed that in W (Figure 3) and IW2 (Figure 4) several individual mice showed serum concentrations beneath the therapeutic level at times, whereas all IW3 (Figure 5) mice tested showed constant high serum concentrations throughout the entire investigation period.

3.2 Experiment 2

3.2.1 Behavioural assessment

3.2.1.1 Drinking behaviour

The pattern of drinking behaviour was not altered in response to surgery. While drinking events during the dark phase occurred frequent and regular, drinking during the light phase appeared rather sporadic and unreliable, comparable to observations acquired in experiment 1 (Figure 1B).

3.2.1.2 Activity analyses

Naïve animals moved a mean distance of 1284±365 cm within 24 h. Compared to naïve animals, distance moved was insignificantly higher after surgery ($F(5, 39)=3.616$, $p=0.772$, Figure 2 A).

3.2.1.3 Body mass, food and water intake

Body mass: Mice weighed on average 23±1.1 g before surgery. No significant difference between treatment groups was found ($F(5, 42)=1.633$, $p=0.438$, Figure 2 B).

Food intake: Naïve mice ate in general 3.6±0.4 g of food pellets a day. Significant differences between groups were found ($F(5, 40)=8.763$, $p\leq0.0001$). Compared with naïve mice, food intake was decreased significantly after surgery ($p\leq0.0001$, Figure 2 C).

Water intake: Naïve mice drank in general 4.8±0.3 g of water a day. Mice did not decrease their water intake significantly after surgery ($F(5, 42) = 1.851$, $p=1.000$, Figure 2D).

3.2.2 Pain scoring

Clinical investigation revealed no physical complications from the surgical procedure performed or of the analgesic treatment, e.g. skin irritation at the injection site, and only minor impairment of animals resulting in clinical investigation scores of 0.13±0.4 at 3 h after surgery was noticed. Narrowing of the orbital area or pulled back ears were rare events seen at 1, 3 and 5 h after surgery (0.81±0.8, 0.69±0.9, 0.13±0.4).

4. Discussion

The aim of this study was to explore whether administering buprenorphine with drinking water offers a reliable treatment option for pain management in mice or whether a combination with buprenorphine injections may be necessary for reliable drug supply. In a first experiment, three protocols were tested in pain free mice: administration via drinking water (W), a combination of two buprenorphine injections during the light phase plus administration via drinking water in the dark phase (IW2) and a combination of three injections during light phase as well as administration via the drinking water for 24 h (IW3). All protocols resulted in mean drug serum concentrations assumed to be therapeutically effective at most of the sampling time points. Nevertheless, blood serum concentrations of individual mice revealed that only the IW3 protocol - continuous supply of buprenorphine with the drinking water and injections every four hours during the light phase – proved to ensure continuous therapeutic blood serum concentrations of buprenorphine in all of the tested pain free mice. Therefore, in a second experiment, IW3 was tested in a standard surgery model and proved to provide prolonged, clinically relevant and reliable analgesia.

Administration of buprenorphine for pain relief by voluntary, oral intake requires a vehicle that is ingested immediately after first presentation and regularly over time. A study in rats by Jessen et al. (Jessen, Christensen et al. 2007) showed that mixing buprenorphine with drinking water induced a significant increase in paw withdrawal latency in the hot-plate-test. However, this latter study also showed that rats tended to decrease their daily water intake when provided with buprenorphine-treated water. Therefore, the first aim of the present study was to assess the willingness of laboratory mice to ingest buprenorphine treated water. The frequency of drinking events as well as the total amount ingested in 24 h was measured. Mice started to drink the buprenorphine treated water immediately and regularly after first presentation, so that no habituation period was needed, and total water intake was not reduced due to buprenorphine administration. This observation of immediate drug intake was remarkable as several authors have reported the need for habituation periods when providing mice with medicated food items (Liles, Flecknell et al. 1998, Kalliokoski, Jacobsen et al. 2011, Hovard, Teilmann et al. 2015). Water intake

appeared frequent and relatively reliable during the dark phase but rather sporadic and less frequent during the light phase due to circadian activity in mice. Drinking pattern was not obviously altered by buprenorphine administration (drinking water and/or injection) or by surgical intervention as seen in the descriptive display of drinking events in figure 1, hinting that voluntary, oral intake may be a possible stress-free administration route for mice.

Nevertheless, due to its first-pass metabolism (Brewster, Humphrey et al. 1981), the efficacy of orally administrated buprenorphine has raised concerns. On the other hand, several studies have shown that buprenorphine has sufficient analgesic efficacy in rodents when administered orally in high concentrations (Liles, Flecknell et al. 1998, Goldkuhl, Jacobsen et al. 2010, Molina-Cimadevila, Segura et al. 2014, Hovard, Teilmann et al. 2015). Moreover, Jessen et al. (Jessen, Christensen et al. 2007) showed that, in rats, buprenorphine administered in drinking water induced antinociception of a greater magnitude and longer duration of action than repeated injections. This possible advantage of the oral over the injection route is also supported by a study performed by Kalliokoski et al. (Kalliokoski, Jacobsen et al. 2011), in which buprenorphine administered with Nutella® led to higher serum concentrations compared to those in mice provided with buprenorphine subcutaneously. In humans, the blood serum concentration of buprenorphine leading to effective analgesia is targeted to be approximately 0.5 ng/mL or higher (Evans and Easthope 2003), whereas in rodents blood serum concentrations of 1 ng/mL are assumed to be effective (Yassen, Olofsen et al. 2005). In our study we observed higher mean and in many cases higher individual blood serum concentrations after oral administration of buprenorphine (1 mg/kg) than after subcutaneous injections (0.1 mg/kg). This could be due to the higher dosage in the drinking water, or it could be that oral administration might have resulted in better absorption and greater bioavailability than the subcutaneous route, as already suggested by Jessen et al. (Jessen, Christensen et al. 2007). Mean blood serum concentrations remained higher than the targeted therapeutic value throughout the whole observation period in W and IW3 animals. In IW2 animals, mean blood serum concentrations remained therapeutic until 4 h after the first injection, but dropped at 6 h after the first injection (18:00) to values below those thought to be therapeutic, while during the drinking water administration in the dark phase, IW2 animals showed high mean serum concentrations until the next day. This shows that buprenorphine provides clinically

relevant serum concentrations, and therefore potential pain relief, only for 4–6 hours after injection, as already suggested by Jirkof et al. (Jirkof, Tourvieille et al. 2015). Therefore, an application interval of 8 h, as commonly practiced, appears too long and might lead to periods with insufficient analgesia in animals undergoing lasting pain—a concern also expressed by others (Gades, Danneman et al. 2000, Carbone, Lindstrom et al. 2012, Jirkof, Tourvieille et al. 2015).

A serious concern in administering drugs with drinking water is the possible reduction of water intake due to surgery and the reliability of providing every individual animal with sufficient analgesia in group housed mice. Jirkof et al. (Jirkof, Cesarovic et al. 2012) showed that mice undergoing a mild to moderate impact surgery like a one-side sham embryo transfer, even when not provided with analgesia, did not decrease their water intake post-surgery. Those animals that had been provided with analgesia, on the other hand, increased their water intake post-surgery, at least during the first six hours after surgery (day phase). In our study mice did neither decrease their water intake post-surgery nor did they considerably change their drinking behaviour, suggesting that mice drank the buprenorphine treated water in sufficient amounts to provide clinically relevant analgesia.

However, most importantly, inter-individual differences in drinking behaviour -namely frequency and intervals of drinking events- led to high variability in serum concentrations of individual mice in four out of five protocols, resulting in periods beneath the targeted serum concentration and therefore potential lack of pain relief.

In consideration of these results, surgery was only performed in combination with protocol IW3, a combination of continuous supply of buprenorphine treated water and three injections of buprenorphine during light phase, which proved to result in continuous, high serum concentrations of buprenorphine. Pain scoring of animals that underwent surgery with the IW3 protocol resulted in low pain scores. In a comparable study, using the same line, sex, housing conditions, surgery techniques and pain indicators animals with saline treatment after surgery reached modified grimace scales of 3.1+/-0.9 and clinical scores of 1.3+/-0.7 one hour after surgery while animals treated with buprenorphine sustained release formulation had scores of 0.7+/-0.4 and 1.1+/-0.9 respectively (Jirkof, Tourvieille et al. 2015). Based on the comparison to these previously observed scores, we assume that the low pain scores, of 0.81±0.8 and 0.13±0.4, assessed one hour after surgery in our study (S), hint on effective pain relief in animals receiving IW3 treatment.

However, in light of our results, caution should be exercised when administering analgesics solely via drinking water in experiments that evoke more than mild pain. In such cases, combined protocols (continuous supply via drinking water plus repeated injections) might be necessary. If stress induced by repeated restraint and injection is of concern sustained release formulations may offer an elegant solution to overcome repeated subcutaneous injections and still provide long-lasting and sufficient pain relief in mice (Foley, Liang et al. 2011, Carbone, Lindstrom et al. 2012, Healy, Tonkin et al. 2014, Jirkof, Tourvieille et al. 2015). In any case, mice need to be monitored closely after painful interventions so that if pain is observed, rescue analgesia, e.g. subcutaneous injections, can be applied.

Regardless of the route of administration, since buprenorphine may have several side effects (increased locomotor activity, and a decrease in food intake and body mass gain), a careful balancing of the impacts of possible side-effects against expected benefits should be performed (Bomzon 2006, Adamson, Kendall et al. 2010, Healy, Tonkin et al. 2014). Jirkof et al. (Jirkof, Tourvieille et al. 2015) showed that home cage activity after injection of a sustained release formulation (2.2 mg/kg body mass) increased significantly and persisted for more than 24 h, resulting in disruption of circadian rhythm, possibly due to the high buprenorphine concentration. In the experiment presented here, all buprenorphine treated animals also showed a tendency of higher home cage activity compared to N animals, with a significant increase in IW3, probably due to continuous high buprenorphine serum concentrations in this group. In the surgery group on the other hand, which had also been supplied with buprenorphine in the drinking water and three injections during light phase this increase in activity was no longer significant, probably due to longer resting periods after surgery. Food intake was reduced significantly compared to N animals, in response to buprenorphine administration (IW2, IW3 and S). We did not observe other side effects, such as abnormal behaviours or sedation, as observed when mice receive high doses of buprenorphine (personal communication M. Guarnieri).

5. Conclusion

In conclusion, administering buprenorphine solely via drinking water may be a pain treatment option in, at least female C57BL/6J, mice. However, sporadic water intake during the light phase and high individual variability in achieved serum concentrations suggests the combination of drinking water plus buprenorphine injections every 4 h in the light phase in order to guarantee continuous therapeutic serum concentrations. In our study the combination of two injections during light phase and drinking water administration during dark phase still appeared insufficient for 24 hour therapeutic serum concentrations, whereas three injections in a four hour interval during the light phase in combination with drinking water administration for 24 h guaranteed continuous therapeutic serum concentrations in all animals. Animals that received this protocol showed very low pain scores after surgery indicating analgesic efficiency of this combination protocol. To overcome concerns of reduced water intake due to pain directly after surgery pre-emptive injections of buprenorphine appear to be an important measure.

Nevertheless, the side effects of buprenorphine, as well as the effects of repeated restraint and injections regarding animal wellbeing and experimental readout must be considered. Such effects might be acceptable when pain relief in experiments with more than mild pain has to be assured and if high buprenorphine intake in individual animals, when combining oral and injection administration, is acceptable. Sustained release formulations might overcome such drawbacks in experiments involving severe or moderate pain in the future.

In experiments involving mild pain only, buprenorphine administration via drinking water alone could be a pain treatment option; however, individual animals could possibly suffer from insufficient pain relief at times.

Therefore, when choosing the appropriate analgesia protocol for mice, a careful balancing of the impacts of possible side-effects against the expected pain impact has to be a mandatory component of animal experiment design.

Acknowledgements

This work was sponsored by grants from the Federal Food Safety and Veterinary Office (Bern, Switzerland) and Society of Laboratory Animals (GV-SOLAS, Germany). The authors would like to thank Robin Schneider, Hugo Battaglia, and the staff of the Central Biological Laboratory for their valuable support as well as Paolo Cinelli for generously providing laboratory resources.

Declaration of conflicting interests

The authors Mareike Sauer, Thea Fleischmann, Miriam Lipiski, Margarete Arras and Paulin Jirkof declare that they have no conflict of interest.

6. Figures & Tables

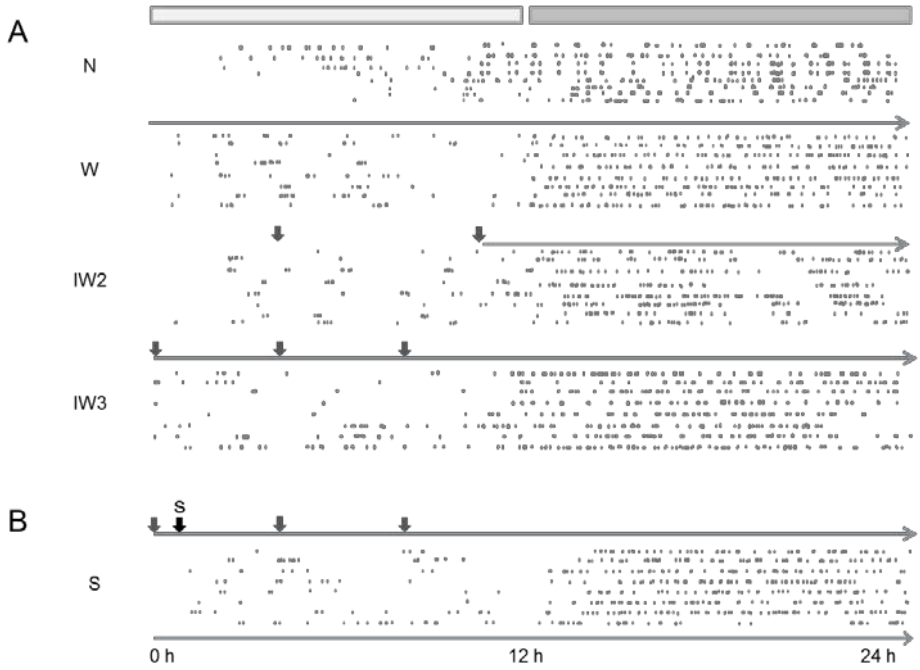

Figure 1: Drinking behaviour over 24 h**:** Water intake was frequent during the dark phase but rather sporadic during the light phase in all groups. Each row of dots represents the drinking events for an individual mouse. Data are solely descriptive, no statistical analysis was applied. **A**: Water intake in naïve (*N*) and with buprenorphine treated (*W, IW2, IW3*), pain free mice. **B:** Water intake in surgically treated mice, provided with three buprenorphine injections during light phase and via drinking water for 24 h. (*N* = Naïve, *W* = Buprenorphine administration via drinking water, *IW2* = Buprenorphine administration via two injections during light phase and drinking water during dark phase, *IW3* = Buprenorphine administration via three injections during light phase and via drinking water for 24h, *S* = surgery plus buprenorphine administration via three subcutaneous injections and drinking water for 24 h.). Dark phase is indicated by a dark grey bar and light phase by a light grey bar. Drinking events are depicted as dots for each individual. Buprenorphine injections are indicated by downward pointing grey arrows, provision of buprenorphine treated water is indicated by

horizontal grey arrows. Surgery time is indicated by a downward pointing black arrow, labeled with an “S”. *n*=8 animals per treatment group.

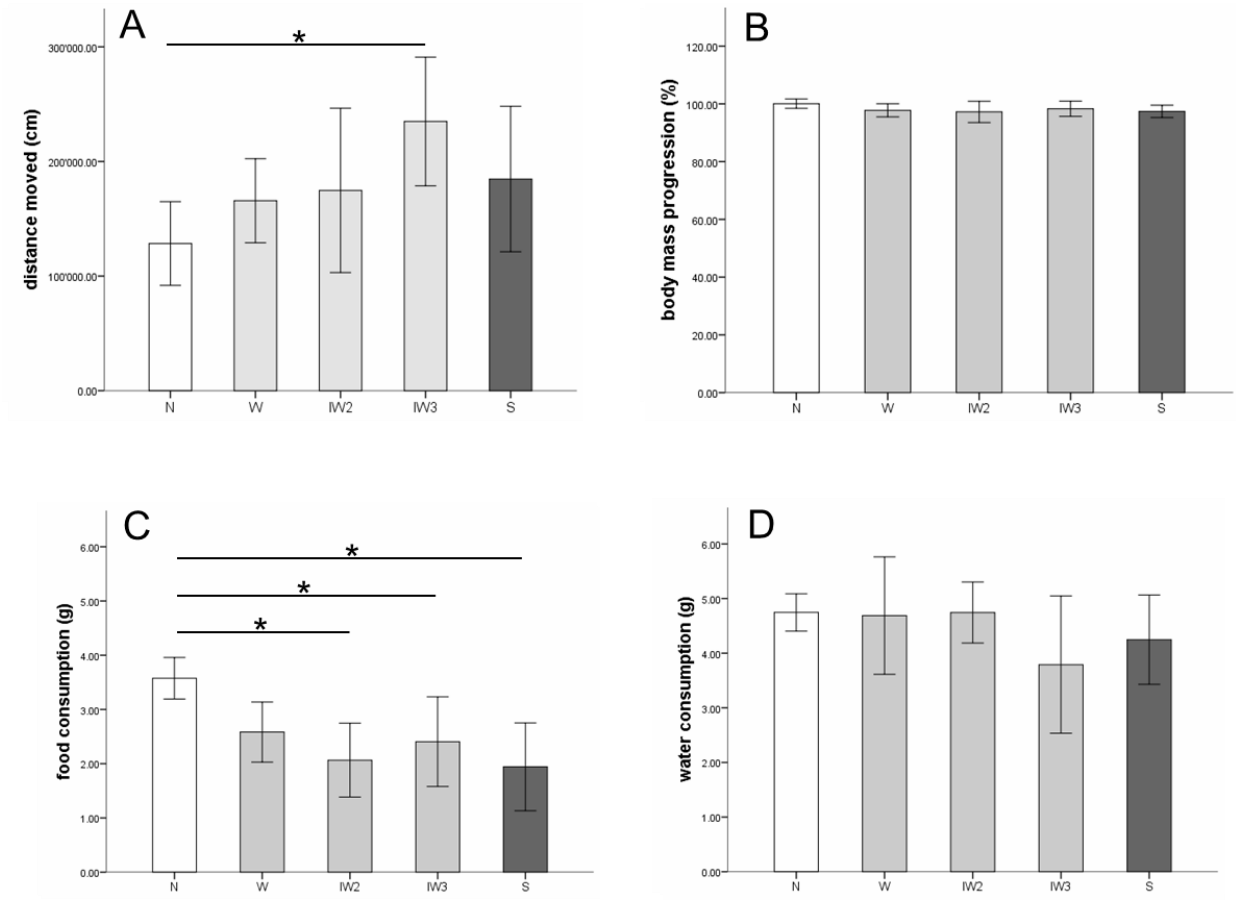

Figure 2: A Activity: Mean (±SD) distance in cm moved during 24h. Distance moved was higher in mice receiving buprenorphine treatment than in naïve mice (p= 0.009); however, these differences were only significant in *IW3* animals (p=0.005). **B Body mass progression:** Mean (±SD) percentage body mass change at 24h after the start of the experiment. No significant differences between groups were found (p=0.172). **C Food consumption:** Mean (±SD) daily food intake in grams. Compared with naïve animals, food intake decreased significantly after buprenorphine administration in *IW2*, *IW3* and *S* treatment groups (*IW2*: p=0.001; *IW3*: p=0.023; *S*= p≤ 0.0001). **D Water consumption:** Mean (±SD) daily water intake in grams. Water intake did not differ significantly between groups (p=0.990).

N = Naïve, *W* = Buprenorphine administration via drinking water, *IW2* = Buprenorphine administration via two injections during light phase and drinking water during dark phase, *IW3* = Buprenorphine administration via three injections during light phase and via drinking water for 24h, *S* = surgery plus buprenorphine administration via three subcutaneous injections and drinking water for 24 h. *n*=8 animals per treatment group. * Significant (p≤ 0.05) differences between experimental groups.

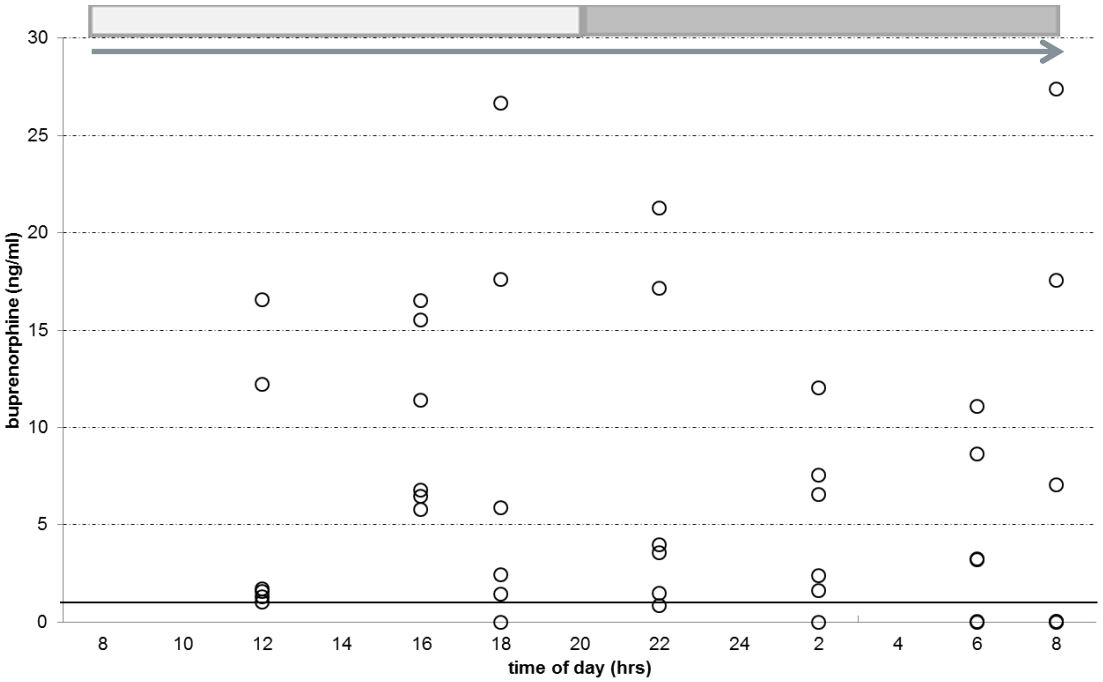

Figure 3: Individual Serum concentrations of buprenorphine in *W* animals at the time points shown. The targeted blood concentration for effective buprenorphine treatment in rodents is 1 ng/ml (indicated by the black solid horizontal line). Provision of buprenorphine in the drinking water is indicated by a horizontal arrow. Light phase is indicated by the light grey bar, dark phase is indicated by the dark grey bar. n=6 animals per time point.

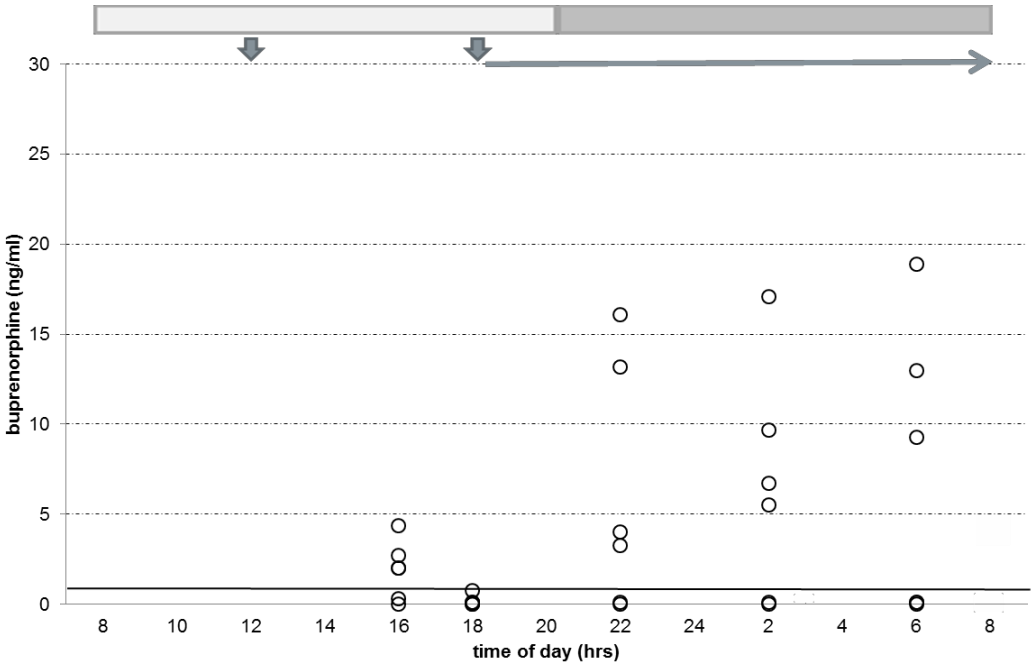

Figure 4: Individual serum concentrations of buprenorphine in *IW2* animals at the time points shown. At 6 h after the first injection (18:00) 90% of the sampled mice show buprenorphine serum concentrations beneath values assumed to be effective.

The targeted blood concentration for effective buprenorphine treatment in rodents is 1 ng/ml (indicated by the black solid horizontal line). Buprenorphine injections are indicated by downward pointing arrows, provision of buprenorphine treated water is indicated by a horizontal arrow. Light phase is indicated by the light grey bar, dark phase is indicated by the dark grey bar. n=6 animals per time point.

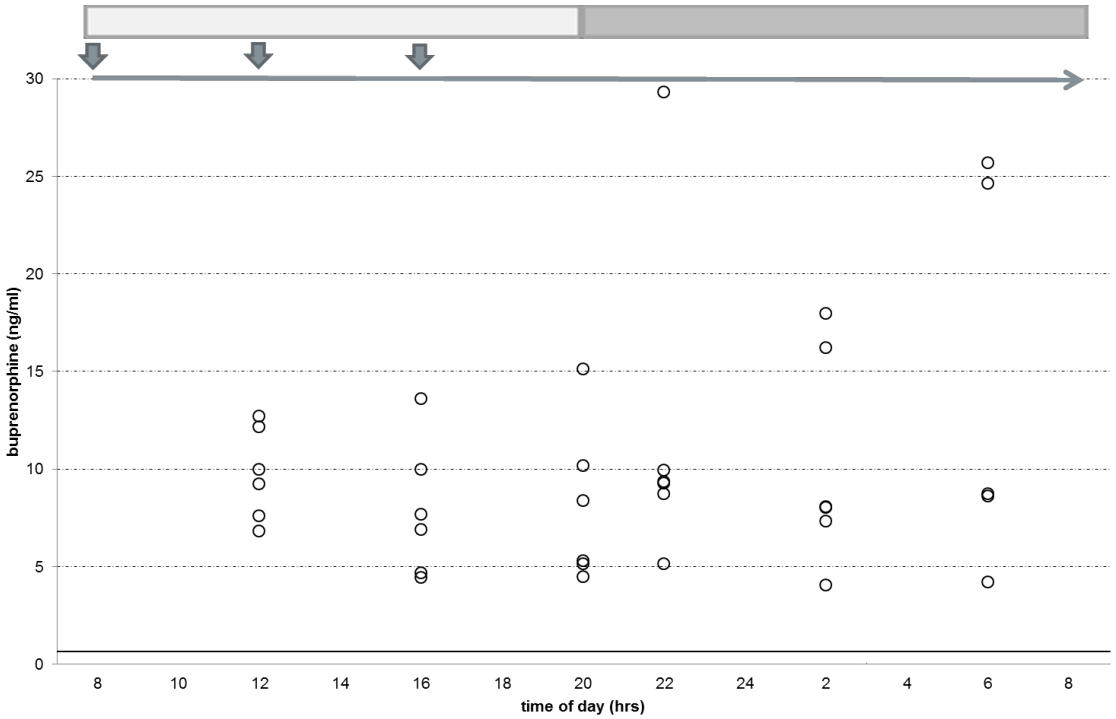

Figure 5: Individual serum concentrations of buprenorphine in *IW3* animals at the time points shown. All mice sampled show buprenorphine serum concentrations assumed therapeutically effective at all time points. The targeted blood concentration for effective buprenorphine treatment in rodents is 1 ng/ml (indicated by the black solid horizontal line). Buprenorphine injections are indicated by downward pointing arrows, provision of buprenorphine treated water is indicated by a horizontal arrow. Light phase is indicated by the light grey bar, dark phase is indicated by the dark grey bar. n=6 animals per time point.

Parameter		Scores
Facial expression		
• orbital tightening • ear position	• narrowing of the orbital area, a tightly closed eyelid, or an eye squeeze (orbital muscles around the eyes contracted) • ears pulled back or rotate outwards and/or back, away from the face. space between the ears may appear wider	not present = 0, moderately = 1, severe = 2
General condition		
• spontaneous behaviour • posture • coat condition • eyes • body condition • wound • movement	• sudden movements, backwards movements, transient involuntary muscular contraction of any body part, kicking with hind paws, licking/biting the wound, highly aggressive, increased vocalization • hunched, arched back, crouched • ruffled, dirty, unkempt, piloerection, hair loss (alopecia) • discharge • sunken flanks, swollen areas, ascites • dirty, bloody, uncleaned, signs of self-injury, signs of inflammation or necrosis, i.e., unusual color (e.g., red, pale) or swollen • apathetic, sedated, decelerated, crawling, immobile, lameness, tiptoe gait	not present = 0, present = 1

Table 1: Scoring system for clinical investigation and behaviour based pain assessment in laboratory mice. In total a max score of 11 can be reached (modified from Jirkof et al. 2015)

Time point / Group	12:00	16:00	18:00	20:00	22:00	02:00	06:00
W	5.72±6.85	10.41±4.77	9.00±10.74	n/a	8.05±8.83	5.02±4.50	4.36±4.57
IW2	n/a	1.9±1.60	0.12±0.30	n/a	6.52±7.61	6.50±6.44	6.86±8.12
IW3	9.77±2.16	7.88±3.17	n/a	8.11±3.73	11.97±7.92	10.29±5.04	17.22±10.36

Table 2: Mean (±SD) serum concentrations of buprenorphine in *W*, *IW2* and *IW3* animals at the time points shown. In *W* and *IW3* animals mean serum concentrations of buprenorphine were higher than the targeted value (1ng/ml) throughout the whole observation period. In *IW2* animals mean buprenorphine serum concentrations decreased beneath the targeted value at one time point: 6 h after the first injection (18.00). n=6 animals per time point.

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Acknowledgements

I would like to express my gratitude to all those who supported me during my work on this thesis, especially to:

Prof. Dr. Margarete Arras, Division of Surgical Research, University Hospital of Zurich, for her exceptional professional guidance and support.

Dr. Paulin Jirkof, for excellent supervision and for sharing her expertise, even if that meant spending the whole night together at the institute.

Dr. Nikola Cesarovic, Dr. Thea Fleischmann, Dr. Miriam Lipiski and Mrs. Flora Nicholls, for always providing help when needed and for always making me smile. It is a pleasure working with you. Special thanks to Miriam for not only being a great colleague and friend but also for being a fantastic work-out buddy.

The wonderful staff at the University Hospital of Zurich and the Biological Central Laboratory, for their kind support and for the excellent work atmosphere.

Last but not least I would like to thank my family, especially my parents Renate and Wigbert Sauer, for their dedication and ongoing support.

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